Photosensitization of Herpes Simplex Virus Type 1 with Neutral Red*

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SUMMARY

Commercial neutral red (NR) originally containing at least 8 components was purified by thin layer chromatography. Herpes simplex virus type 1 (HSV-1) treated in vitro with 3 μg/ml of purified NR became sensitive to light inactivation within 2 min but rapidly lost this sensitivity upon dilution. Similarly, virus grown in the presence of NR lost its photosensitivity upon dilution of the virus stock. In both cases the kinetics of inactivation appeared to be multi-hit. Photoinactivation of intracellular virus was most effective when NR was applied between 6 and 12 h post-infection. The most efficient inactivation occurred when virus at pH 8.8 was irradiated by light at a wavelength of 470 nm.

INTRODUCTION

Herpes simplex virus exposed to heterotricyclic dyes such as neutral red (NR), proflavine and toluidine blue is inactivated in vitro following irradiation by white light (Wallis & Melnick, 1965). Photoinactivation following exposure to NR and methylene blue has been reported to be effective in the treatment of recurrent herpes simplex infections in humans (Felber et al. 1973; Chang & Weinstein, 1975).

Complexes between acridine dyes and nucleic acids cause a wide variety of biological effects. These dyes are intercalated between nucleotide-pairs by extension and untwisting of the phosphate-deoxyribose backbone of DNA, with both the bases and the dye molecules perpendicular to the helix axis (Lerman, 1961, 1963). Guanine moieties are preferentially destroyed after the dye-DNA complex is irradiated (Simon & Vanvunakis, 1962), leading to gaps in the base sequence and subsequent strand breaks in the DNA of the virus (Freifelder & Uretz, 1966). On the other hand, it has been suggested that the failure of Escherichia coli B/r to support the growth of bacteriophage T4 following acridine orange/visible-light treatment results from damage to some part of the bacterial cell membrane in addition to any lesions induced in the DNA (Cramer & Uretz, 1966). Furthermore, irradiation of bacteriophage ϕX174 in the presence of proflavine leads to a loss of serum blocking power suggesting a direct effect of the dye-light treatment on the coat protein (Khan & Poddar, 1974).

Despite its apparent effectiveness, application of dye-light therapy has been severely criticized. It was shown that HSV-1 and HSV-2 inactivated by light irradiation were able to transform cells (Rapp & Jerkofsky, 1973; Rapp & Reed, 1976) and negative clinical
results from placebo-controlled studies on dye-light therapy have also been reported (Myers et al. 1975; Roome et al. 1975; Taylor & Doherty, 1975). In addition, Berger & Papa (1977) have presented circumstantial evidence that the dye-light treatment induced cancer in humans. However, the rationale of the treatment has been effectively defended by Melnick & Wallis (1977). This paper presents evidence indicating that dye-light therapy should be meaningful and suggests how the current therapeutic techniques might be improved.

**METHODS**

**Cells.** Cultures of the BSC-1 line of African green monkey kidney cells were maintained as monolayers on 150 mm Pyrex glass Petri plates in 40 ml of Eagle’s minimal essential medium (MEM-Schwarz-Mann) supplemented with 10% newborn calf serum ('medium') and incubated at 37 °C in a humidified, 5% CO₂ atmosphere. Cells were routinely trypsinized with 10 ml of 0.1% trypsin in phosphate diluent for 10 min at 37 °C and then scraped off the plates with a rubber policeman. Cells were passaged every 4 to 7 days.

**Virus.** The HF strain of HSV-1 was propagated on monolayer BSC-1 cultures grown in 6 oz square glass bottles in 15 ml of medium. The cells were infected with HSV-1 at a multiplicity of infection of 0.01 to 0.1 in 3 ml of medium. The virus was allowed to adsorb to the cells for 3 h with occasional rocking. At the end of the adsorption period an additional 7 ml of medium was added. The yield of virus at 24 h post-infection was 5 × 10⁶ to 5 × 10⁷ p.f.u./ml. Alternatively, cells were infected at an m.o.i. of 0.001 in 4 ml of medium 199 (Schwarz-Mann) with 1% calf serum. Six ml of the same medium was added after 3 h of adsorption. The yield of virus at 36 h after infection was 1 to 5 × 10⁸ p.f.u./ml. The infected cells were stored at 4 °C. To release the virus, a suspension of infected cells was sonicated for 2 min in a sonic bath (Heat Systems Ultrasonic, Inc.).

**Plaque assay.** Monolayer cultures of BSC-1 cells were inoculated with 0.2 ml of virus in MEM, incubated for 2 h and overlaid with 3 ml of agar (0.9% in MEM with 3% calf serum) or starch (prepared as previously described (Fleischmann & Simon, 1973)). At 3 or 5 days, depending on whether starch or agar overlays were used, the plates were stained with NR and the plaques counted.

**Purification of neutral red.** Commercial NR (Matheson, Coleman & Bell Co. Norwood, Ohio) was dissolved in CH₃OH and placed on thin layer chromatography plates made by spreading 21 g of silica gel (Anasil GF, Analabs, North Haven, Conn.) suspended in CHCl₃-CH₃OH (66:3 ml: 33.4 ml) on 3.25 × 4 inch glass plates. Chromatography was carried out with CHCl₃-CH₃ OH (9:2). The commercial NR separated into at least eight components. The brownish red main band (containing about 50% of the material) was removed, extracted with cold 95% alcohol, then dried and the purification process repeated. The final purified product is designated as purified NR. Despite the great degree of purification, purified NR has an absorption spectrum almost identical to that of commercial NR. We were unable to extract the other components of the dye mixture from the silica gel.

**Irradiation of HSV-1 in vitro.** Virus was diluted in phosphate-buffered saline (PBS) containing various concentrations of NR. One ml samples were dispensed into 60 mm plastic Petri dishes, which were then placed on a glow box containing two 15 W cool white fluorescent light bulbs (General Electric) 5 cm from the surface of the box. Virus was diluted and plated immediately after irradiation. Irradiation at specific wave lengths was performed by exposing 2.4 ml of virus in a glass cuvette to the excitation light source of a Perkin-Elmer Fluorescence Spectrophotometer MPF-4. The sample chamber was supplied with a magnetic stirring device to ensure that the sample was constantly stirred during
Fig. 1. Photosensitivity of HSV-1. HSV-1 was diluted 1:10 into PBS (pH 7.3) containing 30 μg/ml of purified NR. One ml samples were taken at 2 min (○—○), 30 min (□—□) and 60 min (△—△). Note that all samples gave essentially identical results. ●—●, Control.

Irradiation thus eliminating problems of self absorption. Light intensities were measured with a Yellow Springs Model 65 Radiometer.

Irradiation of HSV-1 in vivo. Monolayers of BSC-1 cells were infected with HSV-1 at time 0. At various times following a 2 h absorption period, 30 μg/ml of NR was added to each of 2 plates for 2 h. One plate of each set was then irradiated on the glow box for 10 min, following which both plates were washed twice with PBS and re-fed with 2 ml medium. In all cases, the cells were removed from the plates and sonicated to release virus 24 h after infection.

Absorption spectra of neutral red. Commercial and purified NR were diluted in PBS at various pHs and their absorbances measured by the Cary 15 recording spectrophotometer.
RESULTS

Purification of neutral red

Although at least a twofold purification of commercial NR was achieved following thin layer chromatography, the purified material had an absorption spectrum almost identical to commercial NR, and was biologically indistinguishable from it. No photoproduct was detected in a solution of either commercial or purified NR after 60 min of irradiation.

Gain of photosensitivity of HSV-1 following in vitro treatment with neutral red

HSV-1 became sensitive to light inactivation within 2 min following treatment with 30 μg/ml of purified NR in PBS of pH 7.2 (Fig. 1). Ten min of irradiation reduced the titre by over 3 logs. Note the multi-hit inactivation kinetics in this and in Fig. 2.

Removal of neutral red from HSV-1 by dilution

The data of Fig. 2 show that the sensitivities of samples irradiated at 0 min and 60 min after a tenfold dilution from a solution containing 30 μg/ml NR were almost identical to each other and to virus incubated continuously in the presence of the lower concentration of NR. Hence, NR must be lost from the virus within minutes of dilution.
Photosensitization of HSV

Fig. 3. Photoinactivation of HSV-1 in vivo. BSC-1 monolayers were infected with HSV-1 at time 0 at an input multiplicity of 3 to 5 p.f.u./cell. At the indicated times each of two plates were treated with 30 μg/ml NR in Eagle's medium for 2 h. One plate of each pair was then irradiated for 10 min (■—■), the second serving as a control (●—●). The ordinate expresses the yield from irradiated cells 24 h post-infection relative to its unirradiated control. The 0 h point represents the yield from plates treated with NR for 2 h before infection.

In vivo photoinactivation of HSV-1

BSC-1 cells infected with HSV-1 were treated with 30 μg/ml of commercial NR in growth medium at 2 h intervals for 2 h during the growth cycle. Following this treatment, the cells were irradiated and the NR-containing medium replaced by normal growth medium. All plates were sampled 24 h after infection and incubation of irradiated plates beyond 24 h did not increase the yield of virus. In uninfected BSC-1 cells NR was found exclusively in the cytoplasm. However, by 5 h post-infection, most of the NR was in the nucleus. The data of Fig. 3 show that HSV-1 was most sensitive to photoinactivation when the dye was added 6 to 12 h post-infection. The structure of HSV suggested that virus grown in the presence of NR might trap the dye within its nucleocapsid core and thus become irreversibly photosensitive. This was not the case; virus sensitized in vivo still lost its photosensitivity upon dilution. However, the yield of virus from cells treated with 30 μg/ml NR from 3 to 23 h post-infection was tenfold lower than that of control plates (data not shown).

Action spectra of neutral red

Inactivation of HSV-1 was most effective at 430 and 460 nm (Fig. 4). The action spectra of NR at various pHs are shown in Fig. 5. Note that the higher the pH the more susceptible the virus is to photoinactivation. The absorption spectra of NR at various pHs are plotted in the same figure for comparison. The sensitivity of the virus at 430 and 460 nm corresponds to the intensity of absorbance at those wave lengths, while the virus is equally insensitive at all pHs at 530 nm.
Fig. 4. Photoinactivation of HSV-1 at different wavelengths. HSV-1 was diluted 1:10 into PBS (pH 7.3) containing 30 µg/ml of purified NR and samples irradiated as described in Methods. As a control, a non-irradiated sample was put into the cuvette and stirred for varying lengths of time up to 60 min. Under these conditions the incident intensity was 5.8, 6.2, 5.6 and 4.8 x 10⁴ erg/cm²/s at 430, 460, 500 and 530 nm respectively. For comparison, a 1 ml sample of the virus was irradiated on a glow box.

DISCUSSION

HSV has a DNA core, an icosahedral nucleocapsid, and a lipid-glycoprotein envelope. It is rapidly, and reversibly, photosensitized by in vitro treatment with NR (Fig. 1 and 2). The multi-hit inactivation kinetics we observed were reported previously by Rapp et al. (1973). It was also seen by Khan et al. (1977) only after treatment with low levels of proflavine. It is not clear if NR reacts with the virus envelope or with its DNA. In the latter case, the nucleocapsid would have to be permeable to the dye, which is not the case in poliovirus (Wilson & Cooper, 1963), mengovirus (Fleischmann & Simon, 1973) or SV 40 (Rapp et al. 1973) which also have a nucleic acid core encapsidated in a protein coat. These viruses become light-sensitive only after growth in the presence of the dye, and are inactivated with single hit kinetics. On the other hand, a nucleocapsid surrounded by a lipid coat may have a more open structure than one without this protection. These considerations suggest that both the DNA and the coat of HSV-1 might be involved in photoinactivation and this interpretation is supported by the work of Khan & Poddar (1974) who showed that the coat of φX174 can be altered by dye-light treatment.
Irradiation of intracellular virus was most effective when NR was applied between 6 and 12 h post-infection (Fig. 3). This coincides with the period of exponential growth of HSV-1 during replication (Simon & Vanvunakis, 1962; Roizman et al. 1963; Russell et al. 1964). It is possible to explain the data of Fig. 3 by noting that the permeability of the nuclear membrane was apparently altered during the course of the infection. Hence at earlier times NR did not enter the nucleus, where the virus DNA was being made and nucleocapsid assembled, while late in the growth cycle, the dye was entrapped in the nucleus whereas more mature virions were already in the cytoplasm. This may explain why inactivation was relatively inefficient at those times. On the other hand, between 5 and 12 h post-infection, NR in the nucleus could either interact with the virus DNA or bind to the nuclear membrane and become incorporated into the virion when the nucleocapsid budded through it. These results are in contrast to a recent report by Khan et al. (1977). Using a protocol similar to ours, they showed that treatment with a very low level of proflavine 2 to 3 h after infection reduced the final virus yield by a factor of 1000 but that treatment at later times had a progressively lower effect. They also found that production of physical, as well as infective, particles was inhibited.
Oxman (1977) summarizes the reasons for doubting that dye-light therapy could affect recurrent lesions. Principally, it appears that the latent virus is found in sensory ganglia and that the same neurons are involved in each attack. Since new neurons are rarely affected following an attack, he suggests that latent virus remains in the neuron even during an active episode and that therefore local therapy, no matter how effective, cannot alter the course of subsequent infections. However, in view of the positive results of Felber et al. (1973), it is at least possible that all of the latent virions might enter the active lesion and be susceptible to suitable treatment.

The claims that light-inactivated HSV can induce transformation (Rapp & Reed, 1976) fail to distinguish between increasing the frequency of transforming particles in the population (by destroying the ability to form plaques at a greater rate than the ability to transform) and actually converting non-transforming particles into transforming ones. Only in the latter case would the treatment be dangerous. In any event, if NR acts primarily on the virus coat, a conclusion consistent with the multi-hit character of the inactivation curves, then the danger of induced oncogenesis would be insignificant. The presence of an isosbestic point in the absorption spectra of NR suggests that the NR molecule can exist in two different forms, protonated in acidic solution and non-protonated in alkaline solution. Wavelengths of 430 and 460 nm are particularly effective in inactivating the virus-dye complex. Presumably, those wavelengths provide the necessary energy for photodynamic inactivation of the virus by neutral red. A close relationship between the action spectrum and absorption spectrum of NR at pH 8·8 was observed. However, this was not seen at pH 5·9. Although the protonated dye absorbs strongly at 530 nm, this excited molecule is apparently incapable of either exerting a photodynamic effect or of interacting with the virus and inhibiting its replication. The data of Fig. 4 and 5 suggest that using light sources with an output rich in wavelengths of 430 and 460 nm, such as sunlight and blue fluorescent light and applying the NR in an alkaline solution, should improve the efficacy of dye-light therapy in treating recurrent herpes simplex infections.

After this work was completed, publications by Melnick & Wallis (1977) and Jarratt (1977) appeared in which similar observations were reported. These authors suggest that proflavine might be the drug of choice for clinical studies.

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REFERENCES


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